

Fluorescent Protein Tracers

A TRIAL OF NEW FLUOROCHROMES AND THE DEVELOPMENT OF AN ALTERNATIVE TO FLUORESCEIN

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Summary. With the object of simplifying the fluorescent protein tracer technique, the following fluorochromes were examined as possible alternatives to fluorescein: aminoeosin, aminorhodamine B, 3-phenyl-7-isocyanatocumarin (Geigy), 5- β -carboxyethylaminoacridine, R 4388 (Geigy), fluolite C (I.C.I.), lissamine flavine FFS (I.C.I.), lissamine rhodamine GS (I.C.I.), and lissamine rhodamine B 200 (I.C.I.) (RB 200). With the exception of RB 200, none was suitable as a protein label largely because of unsatisfactory fluorescence intensity or colour.

RB 200 has proved a successful alternative to fluorescein. The conjugation of dye to protein by a sulphonamido linkage is quick and simple and does not materially affect the physico-chemical or biological properties of the protein. The resulting conjugates are stable, have a brilliant orange fluorescence in ultraviolet light and good contrast with tissue autofluorescence. The contrast is sufficient to permit the use in microscopy of ultraviolet plus blue light with a yellow filter above the object to ensure a black background; fluorescence is greatly enhanced in this way.

When injected intravenously into rats or rabbits, conjugates are distributed in the tissues and eliminated from the plasma in much the same way as proteins labelled with fluorescein or radio-active isotopes. Serum antibody conjugated with RB 200 retains immunological specificity as demonstrated by the staining of the corresponding antigen. Practical use has been made of RB 200 conjugates as plasma tracers and as specific immunological stains: they have been applied alone and in combination with fluorescein conjugates in double tracing experiments.

INTRODUCTION

THE preparation of serum antibody conjugates with fluorescein isocyanate was described by Coons, Creech, Jones and Berliner in 1942, since which time the method has been improved (Coons and Kaplan, 1950) and the technique and some applications have been reviewed (Coons, 1956). Serum conjugates prepared in this way have been used successfully by many workers, though not as extensively as one might expect, principally, we believe, because the preparation is rather difficult and time consuming. Attempts have been made to overcome some of the difficulties of preparation, e.g. by chromatographic separation of the aminofluorescein isomers (De Repentigny and James, 1954); by adsorption of fluorescein isocyanate on to filter paper from which the conjugation reaction with protein is carried out directly (Goldman and Carver, 1957); by the development of a rapid method for the removal of non-specific staining components in conjugates by extraction with ethyl acetate (Dineen and Ada, 1957). However, none of these modifications has appreciably simplified the method. From time to time claims have been made that

successful fluorescent protein conjugates have been prepared using fluorochromes other than fluorescein, viz. β -anthryl isocyanate, which gives a blue fluorescent conjugate (Creech and Jones, 1941); 1-dimethylaminonaphthalene-5-sulphonyl chloride and nuclear fast red, which give yellow and red conjugates respectively (Clayton, 1954); rhodamine B-isocyanate which gives a red conjugate (Silverstein, 1957). Despite their theoretical advantages, none of these dyes has seriously rivalled fluorescein for general use, sometimes because the conjugations have not been regularly repeatable, sometimes because the fluorescence of the resulting conjugates was unsatisfactory.

This paper is an account of experiments designed to improve the fluorescent protein technique by the development of protein conjugates with fluorochromes other than fluorescein. These experiments eventually led to the preparation of an orange fluorescent conjugate using the fluorochrome, lissamine rhodamine B 200 (RB 200), some details of which have already been described (Chadwick, McEntegart and Nairn, 1958).

METHODS

CHOICE OF FLUOROCHROMES FOR TRIAL

Dyes likely to be useful for the production of fluorescent protein tracers should satisfy the following requirements:

- (1) They should have, or be capable of forming, a chemical group which will react directly with protein to form a stable linkage such as azo, carbamido, or sulphonamido. Examples of such chemical groups are isocyanate, azide, diazonium chloride, and acid chlorides.
- (2) The compound should not possess chemical groups which would interfere with its reaction with protein, e.g. free primary amine groups in the dye could react with the chemical group required for attachment to the protein.
- (3) The intensity of fluorescence should be at least of the same order as that of fluorescein and must not be diminished appreciably by conjugation.
- (4) A fluorescence colour of longer wavelength than fluorescein could be an advantage as this would give better contrast with tissue autofluorescence.

The following nine fluorochromes which satisfied all, or at least the first three of these criteria, were chosen for further investigation: aminoeosin; aminorhodamine B; 3-phenyl-7-isocyanatocumarin (Geigy); 5- β -carboxyethylaminoacridine; R 4388 (Geigy); fluolite C (I.C.I.); lissamine flavine FFS (I.C.I.); lissamine rhodamine GS (I.C.I.); lissamine rhodamine B 200 (I.C.I.). The formulae of these compounds are given in Fig. 1.

PREPARATION OF FLUOROCHROMES IN A SUITABLE FORM FOR CONJUGATION

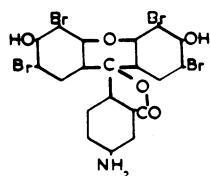
Some of the dyes were ready for conjugation when supplied by the manufacturers, but others had to be modified or prepared in the laboratory.

Aminoeosin was prepared by the reduction of nitroeosin 1 with Raney nickel as for aminofluorescein; the nitroeosin 1 had previously been obtained by the bromination of nitrofluorescein 1 (Cain and Thorpe, 1905).

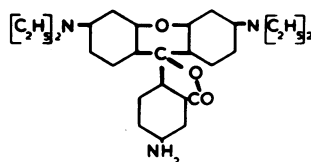
Aminorhodamine B was prepared in a similar way to aminofluorescein. M-diethylaminophenol and 4-nitrophthalic acid were heated together at 180° C. for four hours to

yield nitrorhodamine B, presumably a mixture of the two isomers; no attempt was made to separate the isomers. The mixture was catalytically reduced to the amino derivatives which were used directly for conjugation with protein.

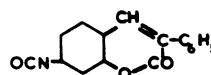
5- β -carboxyethylaminoacridine was prepared by heating 5-chloroacridine with β -alanine and phenol at 120° C. for two hours; on pouring the resultant liquid into ether, the yellow product was precipitated.



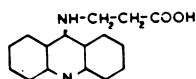
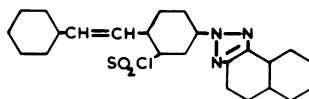
AMINOEOSIN



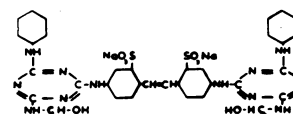
AMINORHODAMINE B



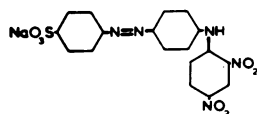
3-PHENYL-7-ISOCYANATOCUMARIN

5- β -CARBOXYETHYLAMINOACRIDINE

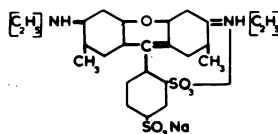
R 4388



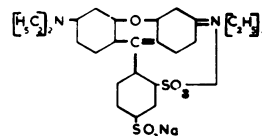
FLUOLITE C



LISSAMINE FLAVINE FFS



LISSAMINE RHODAMINE G S



LISSAMINE RHODAMINE B 200

FIG. 1. Formulae of fluorochromes.

Isocyanates or acid chlorides were prepared as follows:

Isocyanates

The amino derivative of the fluorochrome was treated with phosgene as for amino-fluorescein. The 3-phenyl-7-isocyanatocoumarin compound was conveniently supplied as the isocyanate.

Acid chlorides

Sulphonyl chlorides were prepared by grinding fluorochromes containing sulphonic acid groups, with phosphorus pentachloride as described later for RB 200. The dye R 4388 was supplied as the sulphonyl chloride and therefore only required to be dissolved in acetone before conjugation. 5- β -carboxyethylaminoacridine was converted to the carbonyl chloride by treatment with thionyl chloride.

CONJUGATION PROCEDURE

Most conjugations were carried out with whole rabbit sera, serum albumin or immune serum globulin. Sometimes bovine, goat or horse sera were used. The method of conjugation for the isocyanate group was as described by Coons: the sera were mixed with 0.5 M carbonate-bicarbonate buffer at pH 9, saline, dioxan and acetone, and cooled to 0–2° C. An acetone-dioxan solution of the isocyanate was then added dropwise over a period of 20–30 minutes with continual stirring. Essentially the same procedure was used for the sulphonyl chloride conjugations: an acetone solution of the sulphonyl chloride was added dropwise to the serum mixed with saline and buffer only; dioxan was omitted. For all conjugations, the temperature was kept below 2° C. and stirring was continued overnight (18–24 hr.); any precipitate formed was then removed by centrifugation. Finally, dialysis against regular changes of saline was carried out until fluorescence no longer appeared in the dialysate (5–8 days).

TESTING OF CONJUGATES

The following general scheme was used to test all new conjugates:

- (1) Dialysis followed by treatment with acetone-dried liver powder or activated charcoal to see if conjugation, i.e. the chemical attachment of dye to protein, had in fact occurred.
- (2) Intravenous injection into rats and rabbits to test the biological properties of the conjugates and their stability *in vivo*.
- (3) Examination of immune serum conjugates to ensure that the attachment of dye had not disturbed their immunological properties.

After dialysis, conjugates were examined by ultraviolet light and only if the fluorescence remained brilliant was the conjugation considered to be successful. Such conjugates were treated with liver powder and sometimes with activated charcoal; if their fluorescence was retained, they were considered to be worth further study. Treatment of conjugates with dried liver powder has two functions in the fluorescence technique: (a) the removal of undialysed fluorescent material which is adsorbed on the protein but not chemically attached; (b) the removal of non-specific antibodies. It was used initially by us as an indication of a stable chemical attachment of dye to serum protein. We found, however, that although liver powder did remove considerable amounts of dye (as shown by alcohol extraction of the used powder), it does not remove all the adsorbed dye. Activated charcoal, on the other hand, does remove all adsorbed dye and only chemically attached dye remains after such treatment.

In order to test the biological stability and properties of the conjugates, they were injected intravenously into rats and rabbits at a dose of 40 mg. protein in 0.8 ml. per 100 g. body weight and the test animals were killed 1–4 hr. later. These conditions were the same as those in earlier plasma tracer experiments using fluorescein conjugates which therefore provided a standard of comparison. Unstained formalin-fixed paraffin sections were examined by ultraviolet fluorescence microscopy to locate the fluorescent protein histologically. Full details of the method and equipment have been given elsewhere (Nairn, Chadwick and McEntegart, 1958).

Finally, conjugates which gave satisfactory fluorescence in the tissues of experimental animals were tested as immunological stains. Conjugates of immune rabbit sera were used to stain frozen sections or smears containing the appropriate antigen. The intensity

of specific staining was compared with the intensity produced by the same antiserum conjugated with fluorescein. The immunological specificity of each conjugate was demonstrated by the inhibition of staining produced by pretreatment of the appropriate antigen with unconjugated antiserum. Rabbit antisera, prepared against pneumococcus type III and against a klebsiella (Glaxo 494E), were used for most immunological tests. The technique for the treatment of the tissue sections or smears with the conjugates was as described by Coons and Kaplan.

RESULTS

The following are brief accounts of the fluorochromes examined before the development of RB 200. They failed as alternatives to fluorescein mainly because their fluorescence was insufficiently intense or of a poor contrasting colour. The fluorochromes are arranged according to the chemical group used for conjugation.

(a) Isocyanate Group

(1) *Aminoeosin*. Conjugates gave an intense yellow fluorescence in ultraviolet light, but unconjugated fluorescent material could not be removed by dialysis presumably because it was too strongly adsorbed on the protein. Despite treatment with liver powder, the immune conjugates stained non-specifically. As the fluorescent staining was not bright and its muddy yellowish colour not distinctive, the attempt to obtain a useful conjugate with aminoeosin was abandoned.

(2) *Aminorhodamine B*. When conjugates were treated with liver powder, almost all their colour was removed, although faint specific immunological staining could still be produced with conjugated antisera. An attempt was made to obtain stronger fluorescence by carrying out a second conjugation under different conditions: the phosgene gas was passed through the reaction mixture for a longer time, the concentration of amine during phosgenation was decreased, and the isocyanate solution was added to the serum very slowly. Nevertheless, after treatment with liver powder, the conjugate was pale and gave no better fluorescent staining than before.

(3) *3-phenyl-7-isocyanatocumarin*. Conjugates lost much blue fluorescent material during dialysis but retained sufficient intensity, even after two treatments with liver powder, for successful application as plasma tracers and as immunological stains. However, the intensity of the fluorescence is not as great as that with the corresponding fluorescein conjugates and there is the added disadvantage that the colour is similar to that of tissue autofluorescence.

(b) Carbonyl Chloride Group

5-β-carboxyethylaminoacridine. Conjugates did not lose much of their blue-green fluorescence on dialysis, but unconjugated dye was deposited and this was removed by centrifugation. Treatment with liver powder removed the bulk of the fluorescence from the conjugates, which were then found to have negligible staining properties.

(c) Sulphonyl Chloride Group

(1) *R 4388*. Conjugates gave a dull blue fluorescence colour in ultraviolet light. Although fluorescence was retained after dialysis and treatment with liver powder, its intensity was too poor to justify further examination of the fluorochrome.

(2) *Fluolite C*. The stability of attachment of this blue fluorescent dye to serum protein has been investigated only by dialysis and treatment with activated charcoal. These tests showed that a stable highly fluorescent conjugate can be obtained, but its blue colour limits its value as a fluorescent tracer.

(3) *Lissamine Flavine FFS*. This yielded conjugates with a blue-green fluorescence. When used as direct plasma tracers, they were found to be as intensely fluorescent as fluorescein conjugates, although the colour contrasted poorly with tissue autofluorescence. Conjugated antisera gave intense specific staining but of the same unsatisfactory blue-green colour.

(4) *Lissamine Rhodamine GS*. This gave stable conjugates with a pale orange-yellow fluorescence which were satisfactory both as plasma tracers and immunological stains. Their fluorescence colour is, however, very similar to RB 200 conjugates, whilst their fluorescence intensity is lower.

(5) *Lissamine Rhodamine B 200*. These conjugates have a deep orange fluorescence and are stable; they have been used successfully as plasma tracers and as immunological stains. Since their fluorescence intensity is of the same order as that of fluorescein and their colour contrasts well with tissue autofluorescence, they have been studied in detail. The properties of the dye and its conjugates are described below.

RB 200 AS AN ALTERNATIVE TO FLUORESC EIN

General Properties of the Dye

The dye (Old Colour Index No. 748) is the sodium salt of a disulphonic acid derivative of rhodamine B, and as supplied by the manufacturers is mixed with an equal part of dextrin as diluent. The dextrin is insoluble in acetone and does not appear to interfere with the preparation of the sulphonyl chloride; it is largely removed by the subsequent filtration and has no recognizable effect on the conjugation with protein. Equally successful conjugates have in fact been produced with both the dextrin-free dye and with the commercial product. The dextrin-free dye was obtained by extraction of the commercial dye with ethanol and subsequent evaporation to dryness; the dextrin being relatively insoluble was not extracted. Paper chromatography has shown that the dye contains two components: the red fluorescent material, lissamine rhodamine, present in large amounts, and a slower-moving green fluorescent material present only in traces and therefore unlikely to be important here.

Preparation of RB 200 Conjugates

RB 200 (1 g.) and dry PCl_5 (2 g.) are ground together in a mortar for 5 minutes. Phosphorus oxychloride, produced during this reaction, is volatile and unpleasant, and the grinding is best carried out in a fume cupboard. It is important to use no more than the stated amount of PCl_5 , since an excess may lead to an undesirable acid pH during the conjugation with protein. The PCl_5 used for the production of the sulphonyl chloride should be kept dry; after a fresh bottle has been opened it may be conveniently stored in a desiccator over P_2O_5 . When the dye and the PCl_5 have been thoroughly mixed, 10 c.c. of dry acetone are added and the mixture is stirred gently for a further 5 minutes and filtered. The filtrate is an acetone solution of the sulphonyl chloride of the dye with a deep burgundy colour having a density of the order of a 1 per cent KMnO_4 solution. The dry

acetone is prepared by shaking with CaSO_4 which slowly settles to the bottom of the stock bottle and need not be removed.

Most of our conjugations have been with original serum protein concentrations of about 5 g./100 ml.; we have not found this critical, and successful conjugations have been carried out, using the same volume of buffer and sulphonyl chloride, with concentrations between 1 and 8 g. protein per 100 ml. The protein solution is mixed with an equal volume of physiological saline and an equal volume of carbonate-bicarbonate buffer (pH 9, 0.5M) at 0° – 2° C. and this temperature is maintained during subsequent steps. The saline which was used originally to provide a more convenient bulk does not seem to be essential, as we have latterly had several successful conjugations without it, using only equal volumes of serum and buffer. Nevertheless we prefer this extra dilution when conjugating antisera, to minimise losses of material during subsequent manipulations. The acetone solution of the sulphonyl chloride, 0.1 ml./ml. of original serum, is now added in very small drops over a period of about 15 min. to the buffered serum, which is stirred briskly with an efficient mechanical stirrer but not fast enough to cause frothing. The serum must be kept alkaline throughout the addition of the sulphonyl chloride; this should be checked by spot testing with red litmus paper. If from some technical error, e.g. the use of too much PCl_5 in the preparation of the sulphonyl chloride, the buffered serum becomes acid, satisfactory conjugation may still be achieved by adding more buffer. When the sulphonyl chloride has been added, stirring is continued for a further 15 minutes to complete the conjugation. Originally we continued stirring overnight, but this now seems unnecessary. The temperature for conjugation is not critical: we use 0° – 2° C. to preserve the sera, but have successfully labelled bovine serum proteins at room temperature (18 – 20° C.).

For plasma tracer experiments, the conjugates are dialysed against regular changes of physiological saline until dye no longer appears in the dialysate (6–8 days). They may then be concentrated to the original serum volume by exposing the dialysis sacs to the draught of a fan. Finally, the concentrated conjugate is redialysed against saline overnight to restore normal salt concentration. The colour of the conjugates at this stage is similar to the sulphonyl chloride solution, but with a density approximately that of a 0.1 per cent KMnO_4 solution. Such conjugates contain a small proportion of dye which is not chemically attached to protein but is sufficiently strongly adsorbed to resist prolonged dialysis. This adsorbed dye is no disadvantage for most plasma tracer experiments, as it is quickly detached from the protein and excreted in the urine within a few hours. In acute experiments, e.g. where the animal is killed soon after injection of the conjugated serum, the adsorbed dye may even enhance the fluorescence intensity of the tracer. Where the presence of adsorbed dye is contra-indicated, as in protein plasma clearance studies, it may be removed completely by shaking the conjugate with activated charcoal (0.05 g. per ml.) for 1 hr. followed by centrifugation.

For immunological tracing, treatment with activated charcoal is always desirable and may be used with advantage to replace the 6–8 day dialysis. Overnight dialysis against physiological saline is still required to bring the conjugated serum to the required pH and tonicity. Thus a conjugated antiserum, ready for use, may be prepared within 24 hr. If labelled globulin is required, it is best prepared from conjugated whole serum by precipitation with ammonium sulphate before the final dialysis. A concentrated solution of labelled globulin may be obtained at this stage by dissolving the precipitate in a small volume of physiological saline. Whole serum may be concentrated by evaporation in the

same way as for plasma tracing. Before using the conjugate for immunological staining, treatment with acetone-dried tissue powders is required to remove non-specific antibodies (liver powder is most useful here, 0.1 g./ml. serum, shaken for 1 hr.). It is usually most convenient and economical to treat conjugates with activated charcoal and tissue powders before precipitating the globulins.

Physico-chemical Properties of Conjugates

Using ethanol-purified dye for conjugation with rabbit albumin and crystalline bovine albumin, we have found that 0.04 g. dye reacts with 1 g. of albumin; thus there are about 3 molecules of dye per molecule of protein. A similar figure was obtained by Weber (1952) in some of his conjugations with 1-dimethylaminoaphthalene-5-sulphonyl chloride.

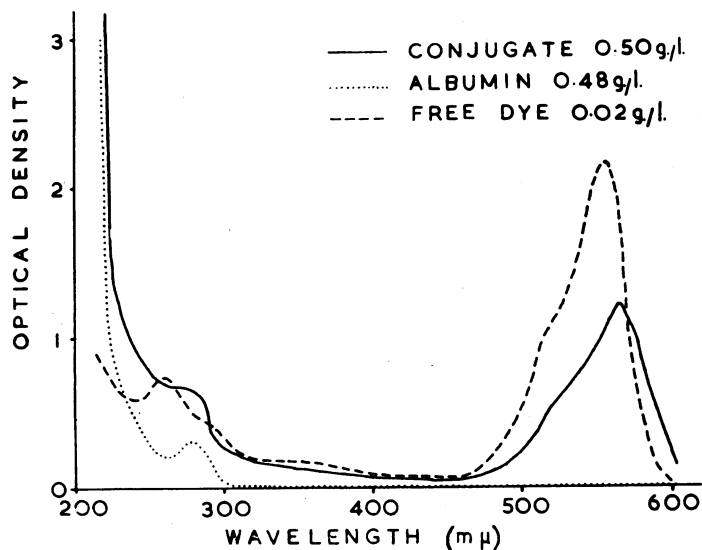


FIG. 2. Light absorptions of RB 200 free dye, bovine albumin and RB 200-conjugated bovine albumin.

This is greater than the figure for fluorescein conjugated with albumin, which Schiller, Schayer and Hess (1952-53) found to be 1.6.

The light-absorptions between 200 and 600 mμ of aqueous solutions of RB 200 free dye, bovine albumin, and RB 200-conjugated bovine albumin have been measured (Fig. 2). The curves obtained for the free dye and the pure albumin are reflected in the shape of the curve of the conjugate, which displays no new characteristics apart from a slight difference in wavelength between its absorption peak and that of the free dye at 560-570 mμ. The fluorescence emissions of the free dye and of the conjugates, measured by the Hartridge spectroscope with an ultraviolet light source, have approximately the same wavelength (610 mμ), although the fluorescence intensity of the conjugate appears to be slightly less than that of the free dye. These observations support the view that the physical properties of the albumin and the dye are not significantly altered by their combination. Another indication that the albumin has not been appreciably changed by the conjugation is shown by comparisons of the electrophoretic patterns of conjugated and unconjugated sera, using an 'EEL' paper electrophoresis apparatus with a barbiturate buffer, pH 8.6.

The patterns of each pair of sera in ten such experiments were not significantly different.

The stability of the conjugates has been investigated: following initial treatment with activated charcoal, they have shown no further loss of fluorescence after dialysis for as long as four weeks; stored at -15° to -20° C., they have remained effective for eight months, the present limit of our experience. However, if conjugates have been stored for more than a week or two, it is often advisable to treat them with charcoal or liver powder before use, as some dye is dissociated from the protein during storage.

Microscopy. In order to obtain maximum fluorescence it is necessary to use a light source of high intensity, such as a high-pressure mercury vapour lamp, and to use as much of the spectrum as possible. In fluorescence microscopy the practicable wavelength range is limited by the need to stop the primary illumination by a filter located above the object; the filter should not diminish contrast between the fluorescence of the tracer and the autofluorescence of the tissue. The orange fluorescence of RB 200 conjugates is sufficiently different from the blue-green autofluorescence of tissues to permit the use of a yellow (blue-stopping) filter above the object. Thus primary illumination by a combination of ultraviolet and blue light up to a maximum wavelength of about $475\text{ m}\mu$ may be used, the yellow filter being sufficient to maintain a black background for microscopy. This light and filter system is unsuitable for fluorescein conjugates, because the yellow filter reduces contrast between their apple green fluorescence and the blue-green of autofluorescence. A colourless or at the most a very pale yellow filter is best for work with fluorescein conjugates and, if a black background is to be obtained, this limits the primary illumination to ultraviolet light only (up to a wavelength of about $410\text{ m}\mu$). The more intense ultraviolet-blue illuminating system used for the microscopy of RB 200 conjugates has definite practical advantages. Not only may preparations be examined in a lighted room without the necessity for dark adaptation, but the location of small amounts of tracer is considerably easier; not least, exposure times for photomicrography are reduced to about one-third. Prolonged exposure of preparations to the intense ultraviolet-blue illumination should be avoided when possible, since it causes fading of the orange fluorescence to a less brilliant yellow.

Biological Properties and Applications of Conjugates

Toxicity. Intravenous injection of the tracer doses of the conjugates has given no sign of toxicity in 22 out of 23 rats and rabbits. One rat developed hypersensitivity oedema immediately after the injection, but this was probably due to the use of heterologous serum rather than to the conjugate *per se*. Injection of equal volumes of the free dye, 0.25 per cent solution in physiological saline, has also been free from toxic effects.

Plasma Clearance. RB 200 serum albumin conjugates, 0.4 g. in 8 ml. per kg. body wt., were injected intravenously into 4 rabbits (2 had homologous albumin and 2 bovine albumin); serum samples were taken at intervals thereafter. The concentration of conjugate in the samples was determined by measuring the light-absorption at the wavelength $565\text{ m}\mu$ (Fig. 3). During the first 48 hours there was a rapid decrease in concentration of conjugate partly due to its distribution throughout the tissues. This was followed by a slower decrease due to metabolism and excretion (the half life appears to be about 5 days); traces of conjugate could still be identified in the plasma after 15 days. This rate of elimination of conjugate from the circulation is faster than the 6.6–9.6 days observed in rabbits given homologous serum albumin labelled with ^{14}C and with ^{131}I (Cohen, Holloway, Matthews and McFarlane, 1956); but it is similar to the rate obtained in rabbits given unlabelled or

^{131}I -labelled heterologous serum albumin (Gitlin, Latta, Batchelor and Janeway, 1951) and in rats given fluorescein-labelled heterologous albumin (Schiller *et al.*, 1952-53). The fate of free dye after injection is quite different. Two rabbits were injected intravenously with 8 ml. per kg. of a 0.25 per cent saline solution of the dextrin-free dye and frequent serum samples were taken. The concentration of dye in the samples was determined as in the preceding experiments except that the light wavelength was 560 m μ . The dye was removed very rapidly from the circulation and its concentration had fallen to 1 per cent of the initial value within 80 minutes; after 24 hours the concentration was too small to be detected.

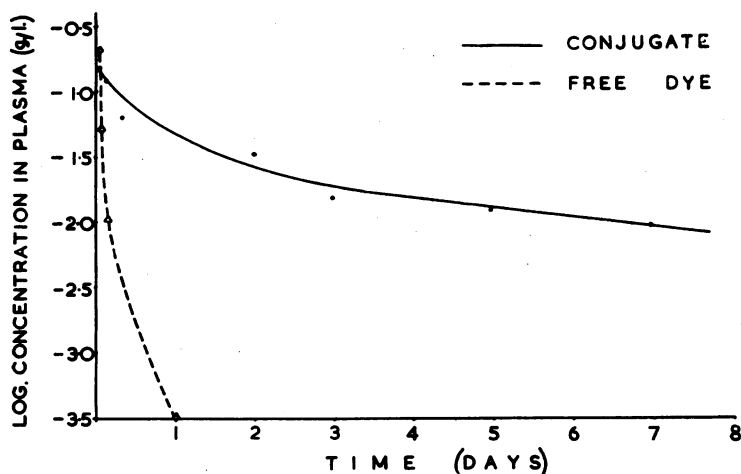


FIG. 3. Rate of clearance from the plasma of a rabbit, of RB 200 conjugated bovine albumin compared with that of the free dye.

Distribution of Conjugates in Vivo. In experimental animals, RB 200 homologous serum or serum albumin is distributed after intravenous injection in the same way as similar proteins labelled with fluorescein. In normal animals the conjugates are found in the plasma (Fig. 4) and concentrated in the Kupffer cells of the liver. Plasma tracer experiments with fluorescein conjugates in pathological conditions (Nairn *et al.*, 1958) have been repeated with RB 200 conjugates and no difference in distribution of the tracer has been observed. Fig. 5 shows the intra-cellular droplets of RB 200-labelled plasma which occur in rabbit liver damaged by the acute experimental hypertension produced by nephrectomy and the injection of renin. It illustrates the good contrast between the fluorescence colour of the tracer and the autofluorescence of the tissue. The contrast is better than that with fluorescein conjugates; smaller droplets of tracer can be detected in the cytoplasm.

Immunological Properties. Antibody globulins prepared against bacterial antigens retained their immunological specificity after conjugation with RB 200. Smears prepared from pure cultures of pneumococci and klebsiella, and frozen sections and peritoneal smears from mice infected with these organisms, could after acetone fixation be stained specifically by the corresponding conjugated antiserum (Figs. 6 and 7). Tissue culture cells infected with representative pox viruses were fixed with acetone or ethanol and treated with the homologous virus antisera labelled with RB 200: staining no less intense than that produced

PLATE I

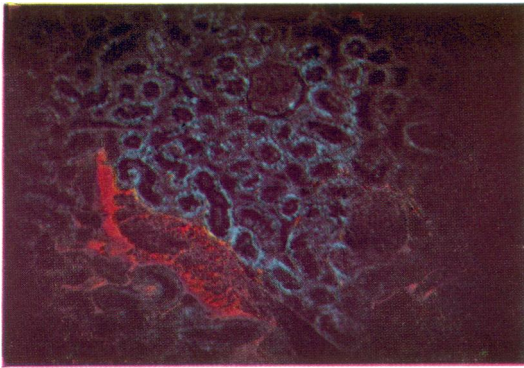


FIG. 4.

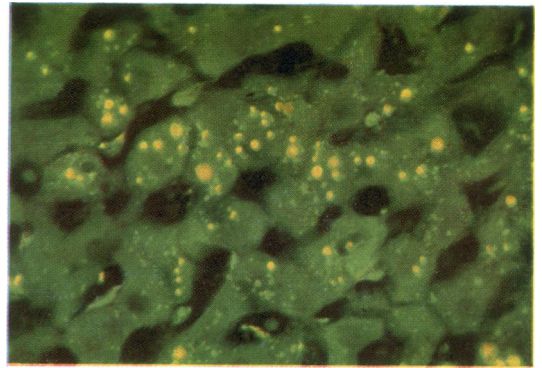


FIG. 5.

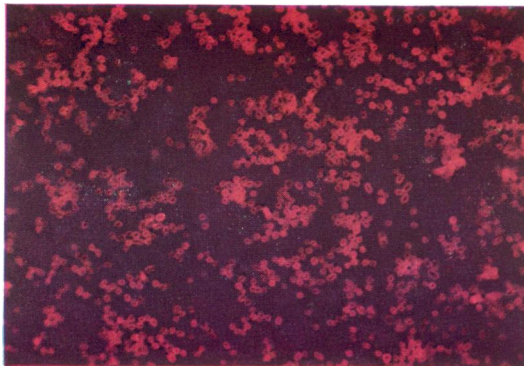


FIG. 6.

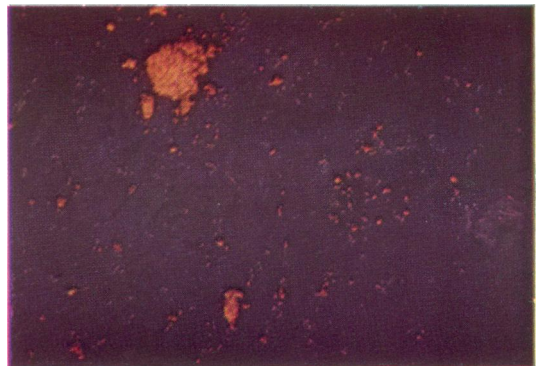


FIG. 7.

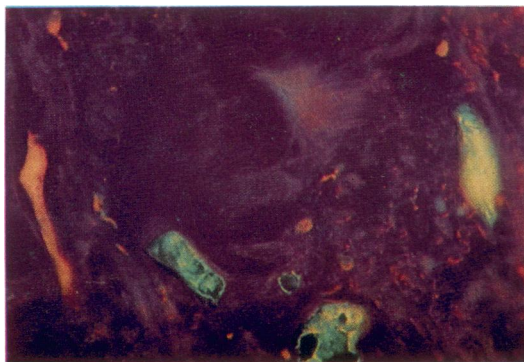


FIG. 8.

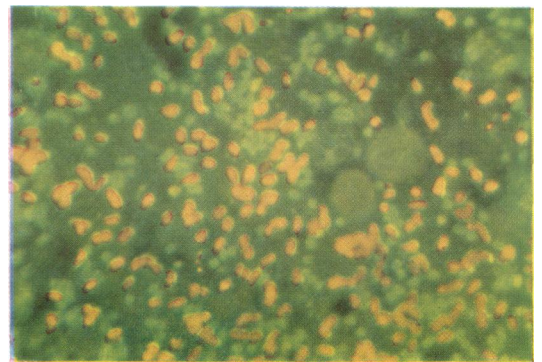


FIG. 9.

Figs. 4-8 are fluorescence photomicrographs from Ektachrome transparencies, Fig. 9 from Agfacolour. The different colour renderings are due mainly to the use of alternative systems of illumination: ultraviolet alone gives the best impression of the deep orange fluorescence of the conjugate; ultraviolet-blue light causes some fading of the tracer during exposure and the yellow filter cuts out the blue component of autofluorescence. Figs. 4, 5 and 8 are examples of direct plasma tracing, and 6, 7 and 9 of immunological tracing. (*See facing page.*)

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by fluorescein conjugates was observed. Antisera to the protozoa *Trichomonas vaginalis* and *Trichomonas foetus* (Belfast and Manley strains) were conjugated with RB 200 and specific staining of acetone-fixed films demonstrated. Antisera to tissue components have been conjugated with RB 200 and successfully applied to the localization of tissue antigens in fresh frozen sections; compared with fluorescein conjugates, such sera have the advantage of better colour contrast with tissue autofluorescence. The globulin from antiserum prepared in a goat against rabbit globulin was conjugated with RB 200 and used successfully in the double layer, or 'sandwich' technique, for the detection of bacterial and tissue antigens.

The success of RB 200 conjugates as immunological tracers does not preclude some loss of titre as a result of conjugation. When attempts were made to see if in fact this had occurred, unexpected difficulties arose. Although labelled pure antibody-globulin is the best material for immunological tracing because it causes least non-specific staining; purified globulin is not always suitable for determining the titre of antibody. We have been unable to obtain precipitation with such globulins and had therefore to estimate the antibody titre of whole antiserum before and after conjugation. The sera used in this way were a rabbit antiserum to whole horse serum, and a rabbit anti-klebsiella serum. The first of these was tested in standard dose against serial doubling dilutions of antigen (Dean and Webb, 1926), and the second in serial dilutions against an agglutinable suspension of the antigen. Several experiments comparing unconjugated serum and conjugates have shown the same general result: there is a loss of serum potency as shown by either precipitation or agglutination titres, but we do not know whether the loss is correlated with a reduction in specific immunological staining. Despite these losses in titre, the sera were successful as immunological stains, but this may have been because the initial titre was high enough to sustain such losses. The reduction in precipitating titre was about 50 per cent, e.g. conjugated antihorse serum tested at a dilution of 1/5 had a precipitating potency for horse serum equal to a 1/10 dilution of the unconjugated control sample. A conjugated anti-klebsiella serum showed a loss of agglutinating titre of about 20 per cent; the antigen was agglutinated by the conjugate to a titre of 1/800, whereas the unconjugated control agglutinated to 1/1000. The problem of the relationship between loss of titre on conjugation and staining potency is complex and requires further study.

FIG. 4. Unstained paraffin section of normal kidney of a rat injected 1 hr. before with RB 200 rabbit serum. Orange fluorescent plasma in a large cortical venule, in some of the intertubular capillaries and in the glomerulus on right, contrasts well with autofluorescence of parenchyma. (Ultraviolet light. $\times 80$.)

FIG. 5. Unstained paraffin section of rabbit liver damaged by acute experimental hypertension. RB 200 homologous serum injected 4 hr. before. Some tracer in the sinusoids and Kupffer cells, but most is abnormally situated as droplets in the cytoplasm of the parenchymal cells. (Ultraviolet-blue light. $\times 370$.)

FIG. 6. Acetone-fixed klebsiella smear treated with RB 200 anti-klebsiella globulin. The coated organisms appear as orange-fluorescent rings. (Ultraviolet light. $\times 370$.)

FIG. 7. Acetone-fixed fresh-frozen section of kidney from a mouse with fatal klebsiella infection. After treatment with RB 200 anti-klebsiella globulin, the section was dehydrated, cleared and mounted in 'Fluormount,' to make a permanent preparation; this has reduced autofluorescence. Organisms brilliantly fluorescent in intertubular vessels and glomeruli. (Ultraviolet-blue light. $\times 120$.)

FIG. 8. Unstained paraffin section of rat subcutaneous tissue showing oedema which developed immediately after intravenous RB 200 heterologous serum; fluorescein-conjugated serum given 90 min. later and animal killed 30 min. afterwards. Orange tracer dispersed in oedema fluid in connective tissue and in lymphatic on left; fluorescein conjugate confined to blood vessels—bottom centre and right. (Ultraviolet-blue light. $\times 370$.)

FIG. 9. Combined pneumococcal, klebsiella infection in mouse. Acetone-fixed peritoneal smear treated successively with RB 200 anti-pneumococcal globulin and fluorescein anti-klebsiella globulin. Organisms coated in contrasting colours; the original orange colour of the pneumococci has faded during photography. Three mesothelial cells with greenish fluorescence right of centre. (Ultraviolet-blue light. $\times 540$.)

The block for this plate was kindly provided by Imperial Chemical Industries, Dyestuffs Division.

Double Tracing. Not only are RB 200 conjugates a useful alternative to fluorescein but they may be used in conjunction with fluorescein conjugates to provide two clearly distinguishable labels. An example of the application of such double tracing is shown in Fig. 8. This is a section of subcutaneous tissue of a rat which developed hypersensitivity oedema following the intravenous injection of RB 200-conjugated heterologous serum; 90 minutes later the animal was given an injection of fluorescein conjugated serum and 30 minutes after this it was killed. The orange conjugate is abundant in the tissue spaces in the protein-rich oedema fluid and its removal by a lymphatic can also be seen; the fluorescein conjugate, on the other hand, is confined to the blood vessels. This experiment provides striking visual evidence that the oedema was associated with leakage of plasma which was transient.

An application of both RB 200 and fluorescein conjugates to immunological tracing is seen in Fig. 9. This is a film of peritoneal exudate from a mouse infected with both a pneumococcus and a klebsiella. The smear, after fixation with acetone, was treated with RB 200-conjugated anti-klebsiella globulin and then with fluorescein-conjugated anti-pneumococcal globulin. The two bacterial antigens are clearly visible in contrasting colours. This method of double immunological tracing was also used to distinguish Belfast and Manley strains of *T. foetus* in mixed films. Although common factors are present in the antisera, it was possible, by cross-absorption of the conjugates, to obtain specific staining of each strain with its own conjugate only.

DISCUSSION

Despite the attractions of the fluorescent tracer technique, the difficulties inherent in the use of fluorescein as a label are a deterrent to more general application of the method. This investigation was undertaken to see if the preparation of tracers could be simplified by means of fluorochromes other than fluorescein. The difficulty in conjugating fluorescein to proteins lies chiefly in the use of the isocyanate group. Special precautions must be taken as the process requires phosgene. Moreover, the isocyanate may undergo secondary reactions during conjugation, e.g. it may react with the hydroxyl groups in the fluorescein molecule, or it may be hydrolysed back to the amine which can then combine with further isocyanate. Such side reactions could partially or completely inhibit the conjugation and might be responsible for some of the failures which occur in practice. For these reasons the isocyanate group may also be unsuitable for the preparation of conjugates with other fluorochromes, and this could be partly responsible for our failure with aminoeosin and aminorhodamine B.

The sulphonyl chloride group might be expected, *a priori*, to be more successful for conjugation than the isocyanate group because it appears to react more rapidly with the amino groups in the protein; the reaction is complete in 30 minutes whereas the isocyanate/protein reaction requires at least 4 hours. A further advantage is that the sulphonamido linkage formed during the conjugation is chemically more stable than the carbamido linkage formed when isocyanate is used. Stability *in vivo* could also be greater because the sulphonamido linkage, unlike the carbamido, is less likely to be affected by naturally occurring enzymes.

Although a number of fluorochromes have been successfully conjugated by means of the sulphonamido linkage, only RB 200 conjugates have suitable fluorescence colour and intensity for practical application. Conjugation with RB 200 has been achieved without material effect on the physico-chemical or biological properties of the protein. The changes in serum antibody following the chemical attachment of the dye have not, however, been

studied in detail. The present experiments indicate that some fall in titre occurs after conjugation, but we do not know if this is due to denaturation of a part of the antiserum by the chemical procedure or to alteration of the antibody proper, as for example by the attachment of dye to the amino groups of the globulin molecule, which might in theory cause blocking of antigen receptors.

The use of RB 200 conjugates offers many advantages. Preparation is quick and easy, requiring no specialized chemical knowledge or equipment; the large volume of conjugate needed for some plasma tracer experiments no longer presents a problem. Furthermore, it is now feasible to carry out double tracing using both RB 200 and fluorescein conjugates in the same experiment. Because of the good colour contrast of RB 200 conjugates with tissue autofluorescence a more intense illuminating system may be used and the tracer detected histologically in smaller amounts than has been possible with fluorescein conjugates; moreover, there is less chance of subjective error in the recognition of specific fluorescence. However, if the potentialities of the method are to be realized a good light source is essential, otherwise even the best conjugates may give disappointing results; under optimum conditions the labelled protein stands out brilliantly in the tissues and should be a valuable new tool in the fluorescent tracer technique.

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